TERMINAL DEOXYRIBONUCLEOTIDYL TRANSFERASE IN HUMAN LEUKEMIA

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Received October 9,1974

SUMMARY

Thymic specific deoxyribonucleotidyl transferase (terminal transferase) was identified and partially purified from peripheral blood leukocytes of a patient with chronic myeloblastic leukemia (CML). The enzyme polymerizes deoxyribonucleoside triphosphates in the presence of an oligodeoxynucleotide alone as initiator, independent of template and the incorporation of one radiolabeled deoxyribonucleotide is inhibited by the presence of other unlabeled deoxynucleotides. The purified enzyme prefers oligo(dA) among various primers tested and favors Mn^{++} for activity. This finding and the previous report in human acute lymphocytic leukemic cells (4) suggest that some human leukemias may be thymic cell derived. Alternatively, the results may be explained by the presence of thymic gland derived cells among the leukemic cells or if in certain leukemias which may have nothing to do with thymus cells, terminal transferase activity is increased.

Terminal deoxyribonucleotidyl transferase (terminal transferase) catalyzes the polymerization of deoxyribonucleoside triphosphate using the 3'-OH end of an oligodeoxyribonucleotide or a polydeoxyribonucleotide as an initiator, independent of template. Terminal transferase was first discovered in calf thymus (1,2). Its function and cellular location are not known. Subsequently, this enzyme was looked for in a variety of tissues but identified only in thymic cells. For instance, it was not found in the bursa of Fabricius of chickens nor in mammalian bone marrow, spleen, lymph nodes, circulating lymphocytes, liver and lungs (3). Recently, McCaffrey et al. (4) showed the presence of terminal transferase in blood leukocytes from 3 of 5 patients with acute lymphoblastic leukemia (ALL) and suggested a thymic origin of the disease. They did not find terminal transferase in HeLa cells, cultured lymphocyte cell lines, and blood cells from a patient with lymphosarcoma and cells from myeloblastic leukemia. The presence of terminal transferase in ALL

cells was confirmed (A. Bhattacharyya, unpublished data) in three cases out of three. Terminal transferase was also identified in peripheral blood leukocytes of two patients with chronic myeloblastic leukemia (CML blastic phase) and two other cases with acute myeloblastic leukemia (AML). The enzyme partially purified from these CML and AML cells has properties similar to terminal transferase from calf thymus and human thymus. In this communication the identification and biochemical characterization of terminal transferase in the cells of a patient with chronic myeloblastic leukemia (CML) with typical markers of CML are reported.

MATERIALS AND METHODS

Source of Leukemic Cells

The patient was a 27 year-old male who had generalized lymphadenopathy and hepatospenomegaly. The morphology of cells from his bone marrow and peripheral smear were typical of CML. Other laboratory findings, diminished leukocyte alkaline phosphatase and presence of the Philadelphia chromosome in marrow and blood cells were also consistent with that diagnosis. Later he went into an acute phase of the disease and his peripheral blood cells were poorly differentiated blasts. These were the leukocytes used for this study.

The whole blood was incubated at 37^{o} for 3 hours. The sedimented red blood cells were discarded and upper layer containing the white blood cells was centrifuged at 600 xg for 10 minutes. The sedimented cells were washed 3 times with RPMI-1640 media salt solution (Grand Island Biological Co.). The cells were suspended in the same solution containing 10% (v/v) dimethyl salfoxide and were kept frozen at $-70^{o}C$.

Human Thymus

The thymus was obtained during cardiac surgery of a three year-old girl.

Preparation of Crude Extract

Forty gms of cells were washed twice with PBS and then suspended in 80 ml of buffer containing 50 mM Tris-HCl (pH 7.9), 20 mM dithiothreitol (DTT),

5 mM $MgCl_2$ and 0.2 M sucrose. The suspension was dounced manually in a glass homogenizer (teflon pestle) until 90-95% cells were broken (monitored by taking an aliquot and examining under phase contrast microscope). The nuclei and mitochondria were separated by differential centrifugation first at 1000 xg for 10 min. and then at 10,000 xg for 15 min. The postmitochondrial supernatant was placed on top of a 3 ml of 25% (w/w final) sucrose cushion in homogenizing buffer and centrifuged at 150,000 xg for 2 hrs. in a type 65 rotor (Beckman). The supernatant was aspirated out and saved.

The high speed pellet (150,000 xg pellet) was suspended in 50 mM Tris-HCl (pH 7.9) containing 1 mM DTT and made to 0.8 M KCl and 0.5% triton X-100. The mixture was stirred at 0-2^{o}C for 2 hrs. Then it was centrifuged at 100,000 xg for 1 hr. The supernatant was taken and the pellet was reextracted as before. The supernatants were pooled and dialyzed against 50 mM Tris-HCl (pH 7.9), 1 mM DTT and 20% glycerol (v/v) (buffer A). The dialyzed material was used as the starting crude extract for further studies.

The nuclear pellet and the mitochondrial pellet were processed exactly in the same way as above.

The 150,000 xg supernatant was made 70% saturation with $(NH_4)_2\ SO_4$ and the precipitate was dissolved in 50 mM Tris-HCl (pH 7.9) containing 1 mM DTT and dialyzed against 100 volumes of buffer A with 2 changes of buffer. The concentrated and dialyzed materials was used as crude extract.

Human thymus was also processed in the same way as described above.

Estimation of Terminal Transferase

An aliquot (0.4 ml) of the crude extract from the high speed pellet was layered on top of a 20-60% glycerol gradient in 50 mM Tris HCl (pH 7.9) and 1 mM DTT and was centrifuged at 40,000 rpm in a SW-41 rotor (Beckman) for 16 hrs. The gradient was fractionated into 27 to 30 equal fractions and assayed for terminal transferase activity as described in Table 1. The units of enzyme activity per gm of tissue were calculated by summing the activity on the gradient.

Terminal transferase contents were also estimated from the other

Table 1

Content of Terminal Transferase in Human

Leukemic Cells and Normal Human Thymus

Tissue	Units of Terminal Transferase per gm of Tissue				
	Nucleus	Mitochondria	High Speed Pellet (150,000 xg pellet)	Soluble Supernatant	Total
Human CML Blast Cells	9.0	0.2	11.8	13.2	34.2
Normal Human Thymus	17.1	0.3	24.6	35.3	77.3

A portion of the crude extract (0.4 ml) was layered on top of a 20-60% (v/v) glycerol gradient and was spun at 40,000 rpm for 16 hours in a SW-41 rotor (Beckman). The gradients were fractionated into 27 to 30 equal fractions. 15 µl from each fraction were taken and assayed for terminal transferase. The reaction mixture contained 50 mM Tris-HCl pH 7.9, 50 mM KCl, 5 mM DTT, 0.6 mM $MnCl_2$, 50 µg/ml of dA_{12-18}, 35 µM ^{3}H-dGTP (sp. ac. 13 Ci/mM), 500 µM cold dGTP and the enzyme. The reactions were for 1 hour at 37°C. The reactions were stopped by adding 2 ml of 10% CCl_3COOH. The samples were washed on millipore filter paper with 5% CCl_3COOH, dried and counted in liquid scintillation counter. The total units of the enzyme was calculated by summing the activity on the gradient. One unit of the enzyme being the amount of enzyme which incorporates 1 nM of dGTP per hour into acid insoluble polymer. All crude extracts were processed under identical conditions.

subcellular fractions of the leukemic cells and normal human thymus (see Table 1).

Purification of Terminal Deoxyribonucleotidyl Transferase

The crude extract as described above was applied on a DEAE-cellulose (DE-52) column (25cm x 2cm) preequilibrated with buffer A. The column was washed with buffer A and the enzyme was eluted with 50 mM NaCl (approximately 75% of the input activity was eluted). The eluted enzyme was pooled, dialyzed and loaded on a phosphocellulose (P-11) column (10cm x 1cm) preequilibrated with buffer A. After washing the column with buffer A, the enzyme was eluted with 0-0.6 M linear NaCl gradient. The activity eluting at salt concentration

of 0.36 to 0.39 M was pooled, dialyzed and concentrated by dialyzing against buffer A containing 50% glycerol (v/v). This was then further purified in a 10-30% (v/v) glycerol gradient containing 50 mM Tris-HCl (pH 7.9), 1 mM DTT

Table 2

Requirements of the Terminal Transferase

From Human Leukemic Cells (CML)

Addition	pmole of ^{3}H-dGMP incorporated per 10 µg of enzyme
Complete + Mn^{++} + dG_{12-18}	288.0
Complete + Mn^{++} + dG_{12-18}·rC	244.8
Complete + Mg^{++} + dG_{12-18}	77.7
Complete + Mg^{++} + dG_{12-18}·rC	69.9
Complete + activated DNA + Mn^{++}	1220.9
Complete + activated DNA + Mg^{++}	1090.8
Complete + activated DNA + Mn^{++} + dATP	258.5
Complete + activated DNA + Mn^{++} + dATP + dCTP	217.8
Complete + activated DNA + Mn^{++} + dATP + dCTP + dTTP	190.7
Complete + dG_{12-18}	2.1
Complete + Mn^{++}	2.3
Complete + dG_{12-18} + Mn^{++} Minus enzyme	2.5

The complete reaction mixture contained 50 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM DTT, 0.05% Triton X-100, 35 µM ^{3}H-dGTP (sp. ac. 13 Ci/mM), 500 µM cold (non radioactive) dGTP, 50 µg/ml of primer and 15 µl of enzyme in a total volume of 50 µl. The concentrations of Mn^{++} and Mg^{++} were 0.6 mM and 10 mM respectively. The concentration of unlabeled nucleotides were 550 µM. Reactions were for 60 min and the samples were processed as in Table 1. Protein was estimated according to the method of Lowry et al.

and 0.5 M KCl. The gradients were centrifuged at 40,000 r.p.m. for 36 hrs. in SW 41 rotor (Beckman). Ovalbumin was used as a marker. The gradients were fractionated and assayed for activity as in Table 1. The peak of the activity was pooled, dialyzed against buffer A and was used as the source of the enzyme. The enzyme has a molecular size of 3.3S. In such preparations DNA polymerase of any kind could not be detected (Table 2).

RESULTS

Estimation of Terminal Transferase

Terminal transferase was estimated in the subcellular fractions of the CML cells and the normal human thymus. All subcellular fractions contained

Table 3

Primer and Substrate Preferences of Terminal Transferase from Human Leukemic Cells

Primers	pmole of Nucleotide Incorporated/10 µg of enzyme			
	dAMP	dCMP	dGMP	dTMP
dA_{10}	191.7	561.7	1345.4	205.0
dC_{10}	35.9	86.1	392.6	35.0
dG_{10}	62.5	58.1	280.0	28.8
dT_{10}	34.3	28.4	191.5	11.9

The reaction mixture contained 50 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM DTT, 0.05% Triton X-100, 0.6 mM $MnCl_2$, 50 µg/ml of primer, 535 µM deoxyribonucleotide and the enzyme. 535 µM deoxyribonucleotide represents 500 µM cold deoxyribonucleotide and 35 µM radioactive (same) nucleotide. The concentrations of the substrates and the primers were in the plateau of the concentration curve (data not shown). The reactions were for 30 min. and the samples were processed as described in Table 1. Oligo or poly ribonucleotides were not used as primers (data not shown).

this activity but the soluble supernatant contained the highest activity (Table 1). With these procedures the CML cells reported here contained 34.2 units of the enzyme per gm of cells compared to 77.3 units per gm of the normal human thymus tissue. These typical CML cells contained unusually high amounts of the enzyme compared to cells obtained from many other types of leukemia. Usually 4 to 10 units of the enzyme per gm of cells were found in ALL (3 cases) and CML (2 cases in acute blast phase) (unpublished data).

Requirements of the Terminal Transferase

The terminal transferase purified from these CML cells can polymerize any deoxyribonucleoside triphosphate using an oligodeoxynucleotide or a polydeoxynucleotide alone as an initiator, independent of template. The enzyme can also polymerize a single deoxyribonucleotide when activated DNA was used as initiator (Table 2). The polymerization of a single radiolabeled deoxyribonucleotide is strongly inhibited by the presence of other cold (non-radioactive) deoxyribonucleotides (Table 2). The polymerization of a deoxyribonucleotide using an oligodeoxynucleotide an initiator is not enhanced when a complementary template strand was supplied. These results suggest that the enzyme is a true terminal transferase and is free from any true DNA polymerase activity (Table 2). The polymerization of deoxyribonucleotides is dependent on the free 3'-OH end of an oligo or polydeoxynucleotide. Ribo oligomers or ribo polymers are very poor initiators, and we find that under our optimum conditions for assay that the enzyme prefers Mn^{++} to Mg^{++}. The terminal transferase activity is completely inhibited by a combination of 10% ethanol and 10 mM N-ethylmaleimide while under this condition the DNA polymerase β is not inhibited. Therefore, the terminal transferase activity is not due to a minor terminal addition of deoxyribonucleotides catalyzed by DNA polymerase β. The enzyme prefers oligo dA irrespective of substrates. A comparison of the preferences of primer and substrates of the terminal transferase is shown in Table 3.

DISCUSSION

Terminal deoxyribonucleotidyl transferase was thought to be

thymic specific since the enzyme was not found in a wide variety of tissues (3). McCaffrey et al. (4) found terminal transferase in the leukocytes of patients with ALL and suggested a possible thymic origin of the disease. We have found terminal transferase in human ALL cells confirming the results of McCaffrey et al. (4). Srivastava has shown the presence of terminal transferase in a variety ofcells (5) and has demonstrated that the amount of terminal transferase in normal cells are 0.2 to 1% as compared to leukemic cells (personal communication)

The results presented in this communication clearly show the presence of terminal transferase in human CML (blastic phase) cells. These results may be interpreted as indicating one of the following: (a) that terminal transferase may be thymic specific but may be derepressed in other tissues possibly from expression of type-C viral information (6,7) or by the neoplasia itself; (b) that the stem cells of many human leukemias are in fact thymic derived; or (c) that there are thymic cell responses to the disease so that leukemic cells are contaminated with cells derived from the thymus gland but are not themselves thymic derived. In this respect it will be of interest to show by a histochemical assay that it is the leukemic cells which contain this enzyme and that the amount is comparable to activities found in thymus cells.

ACKNOWLEDGEMENTS

The leukemic cells were generously supplied by Dr. H. G. Braine of Baltimore City Hospital, Baltimore, Maryland and Dr. R. C. Gallo, of NCI, NIH, Bethesda, Maryland. The human thymus was kindly supplied by Dr. Andrew Morrow of NIH, Bethesda, Maryland. Thanks are due to Mr. S. A. Hasan for excellent technical assistance. This work was supported in part by Contracts 1-CM-12340 of the Cancer Treatment Area and 1-CP-332111 of Virus Cancer Program of National Cancer Institute.

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